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# Embryonic stem cell-derived hematopoietic stem cells

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Despite two decades of studies documenting the *in vitro* blood-forming potential of murine embryonic stem cells (ESCs), achieving stable long-term blood engraftment of ESC-derived hematopoietic stem cells in irradiated mice has proven difficult. We have exploited the *Cdx-Hox* pathway, a genetic program important for blood development, to enhance the differentiation of ESCs along the hematopoietic lineage. Using an embryonic stem cell line engineered with tetracycline-inducible *Cdx4*, we demonstrate that ectopic *Cdx4* expression promotes hematopoietic mesoderm specification, increases hematopoietic progenitor formation, and, together with *HoxB4*, enhances multilineage hematopoietic engraftment of lethally irradiated adult mice. Clonal analysis of retroviral integration sites confirms a common stem cell origin of lymphoid and myeloid populations in engrafted primary and secondary mice. These data document the cardinal stem cell features of self-renewal and multilineage differentiation of ESC-derived hematopoietic stem cells.

Cdx4 | clonal analysis | HoxB4

Transplantation of bone marrow (BM)-derived hematopoietic stem cells (HSCs) is the standard treatment for high-risk leukemia and a range of genetic disorders of the blood. However, a shortage of HLA-matched BM donors and the inability to culture and genetically repair BM-derived HSCs *in vitro* have limited more widespread therapeutic applications (1). When generated by somatic cell nuclear transfer, pluripotent embryonic stem cells (ESCs) provide a theoretically unlimited source of autologous hematopoietic progenitors and an alternative strategy for treating leukemia and genetic bone marrow disorders (2, 3). Although ESCs can differentiate into all lineages of the blood system *in vitro*, efficient production of functional HSCs that can reconstitute all hematopoietic lineages *in vivo* has proven difficult (4).

One approach to obtain definitive HSCs from ESCs is to enforce expression of genes that stimulate hematopoiesis or enhance HSC function. The homeodomain gene *HoxB4* has been shown to enhance competitive engraftment of murine BM-HSC and induce proliferation of progenitors from human cord blood without inducing leukemia, thereby making *HoxB4* an excellent candidate gene for our studies (5–11). Previously, we successfully engrafted lethally irradiated mice with ESC-derived hematopoietic progenitors engineered to ectopically express *HoxB4*. When introduced into hematopoietic precursors dissected from the precirculation murine yolk sac, *HoxB4* promoted long-term multilineage engraftment, suggesting that this homeodomain gene helped specify definitive hematopoietic fate from primitive hematopoietic progenitors (12). However, the extent and durability of lymphoid engraftment from either ESCs or yolk sac populations was minimal in these engrafted animals, possibly due to the inability to fully pattern definitive HSCs from these embryonic populations.

Our understanding of how *Hox* genes promote hematopoietic specification has been greatly advanced by insights into the role of *Cdx4*, which along with *Cdx1* and *Cdx2* represent a family of caudal-related homeobox-containing transcription factors that specify posterior tissue fates and mediate anterior-posterior patterning through modulation of *hox* gene expression (13–15). *Cdx4* was shown to be necessary for blood formation in the zebrafish and to promote hematopoietic colony formation when ectopically ex-

pressed in ESCs (16). *Cdx4* null zebrafish have reduced expression of hematopoietic genes, including *SCL*, *Runx1*, and *GATA1*, whereas overexpression of *Cdx4* induces ectopic blood formation and alters *Hox* gene expression patterns, including up-regulation of *HoxB4* (16). *Cdx1* functions redundantly to promote blood formation in zebrafish (Alan Davidson, personal communication). *Cdx2* is a translocation partner of *TEL* (*ETV6*) in human acute myeloid leukemia (17), and overexpression of *Cdx2* alone results in transplantable acute myeloid leukemia in a mouse model (18). These findings suggest that a genetic pathway involving *cdx* and *hox* genes plays an essential role in blood formation and provide a central mechanism for driving hematopoietic specification from ESCs.

In this study, we have explored the effect of *Cdx4* expression on hematopoiesis in the murine ESC system. Using a murine ESC line with tetracycline-inducible *Cdx4*, we demonstrate that *Cdx4* promotes commitment to hematopoietic mesoderm, stimulates hematopoietic progenitor formation from ESCs, and promotes lymphoid potential of ESC-derived HSCs. Using ESCs engineered to ectopically express both *Cdx4* and *HoxB4*, we demonstrate radioprotection and robust and stable engraftment of hematopoietic lineages in irradiated mice. Moreover, we apply proviral integration analysis in fractionated myeloid and lymphoid lineages of primary and secondary mice to document the clonal derivation of self-renewing, multipotential HSCs from ESCs.

## Methods

**Cell Culture.** ESCs were maintained and differentiated according to published protocols in ref. 12. Doxycycline was added to the culture medium from day 3 to day 4 at 0.1  $\mu\text{g}/\text{ml}$  and from day 4 to 6 at 0.5  $\mu\text{g}/\text{ml}$  to induce *Cdx4* expression. Cells were harvested at day 6 by collagenase treatment. A total of  $10^5$  embryoid body (EB) cells were plated onto semiconfluent OP9 cells in six-well dishes and were infected with retroviral supernatants, produced in 293 cells by Eugene (Roche) cotransfection of viral plasmid MSCV-HoxB4-ires-GFP and packaging-defective helper plasmid, pCL-Eco. Infected EB cells were cultured according to protocols in ref. 12. Blast colony forming/replating assay and hematopoietic colony formation assay were performed as described in refs. 19 and 20.

**RT-PCR Analysis and Quantitative Real-Time PCR.** Cells were harvested in RNA Stat-60 (Tel-Test), and total RNA was isolated. All RNA samples were treated with DNaseI and purified by RNeasy MinElute kit (Qiagen). cDNAs were prepared according the manufacturer's instruction (Invitrogen). Real-time PCR was performed in triplicates with TaqMan reagent kits (Applied Biosystems) on an ABI Prism 7700 Sequence Detector. GFP DNA levels were quantified into arbitrary units by using the comparative  $C_T$  method (relative to the TDAG51 gene as an internal normalization control) (21). For Fig. 1 *E* and *F*, test gene expression was normalized to

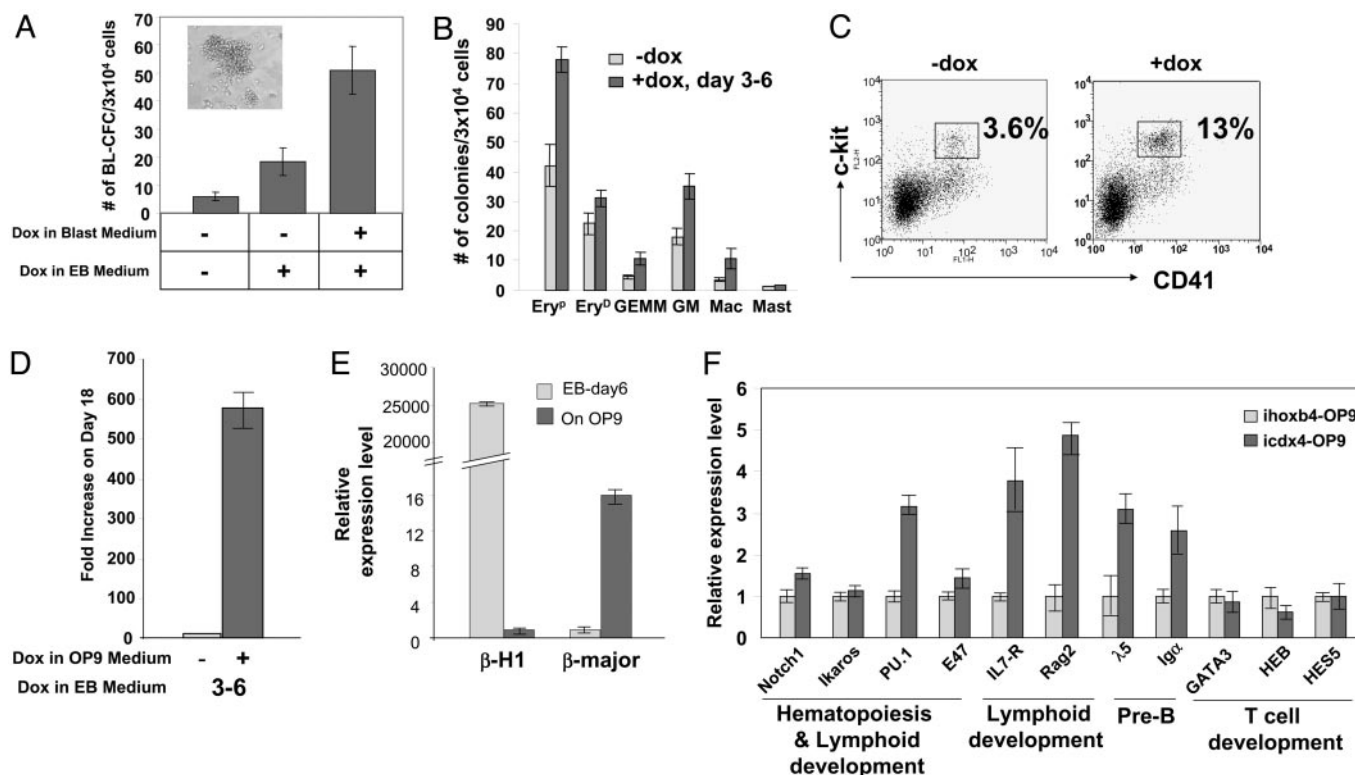
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Abbreviations: BM, bone marrow; CFU-S, colony-forming units of the spleen; EB, embryoid body; HSC, hematopoietic stem cell.

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**Fig. 1.** Characterization of ESC-derived hemangioblast and hematopoietic progenitors from an inducible *Cdx4* cell line. (A) Quantification of blast colony-forming cells (BL-CFCs). A total of  $3 \times 10^4$  EB cells harvested on day 3.2 of differentiation from an inducible *Cdx4* cell line were plated in blast-colony forming media in the absence or presence of doxycycline (dox), and colonies were counted 4 days after plating. A photograph of a representative blast colony is shown (Inset). (B) Methylcellulose colony-forming potential of day 6 EB-derived cells plated in methylcellulose containing cytokines (M3434). Colonies were counted from day 5 to 10 after plating. Ery<sup>P</sup>/Ery<sup>D</sup>, primitive/definitive erythroid; GEMM, granulocyte, erythroid, macrophage, megakaryocyte multilineage; GM, granulocyte macrophage; Mac, macrophage; Mast, mast cell. (C) Flow cytometric analysis of c-kit and CD41 on day 6 EBs. (D) Inducible *Cdx4* ESC were treated with doxycycline from days 3 to 6 of EB formation and cultured on OP9 cells in the absence or presence of doxycycline. Fold increase of cell number on day 18 of OP9 culture was calculated relative to the initial cell number. (E) Relative expression levels of fetal ( $\beta$ -H1) and adult hemoglobin ( $\beta$ -major) before and after OP9 expansion by real-time RT-PCR analysis. (F) Relative expression levels of genes specific to different hematopoietic and lymphoid development pathways in *Cdx4*-induced or *HoxB4*-induced ESC-derived hematopoietic progenitors 15 days after OP9 expansion.

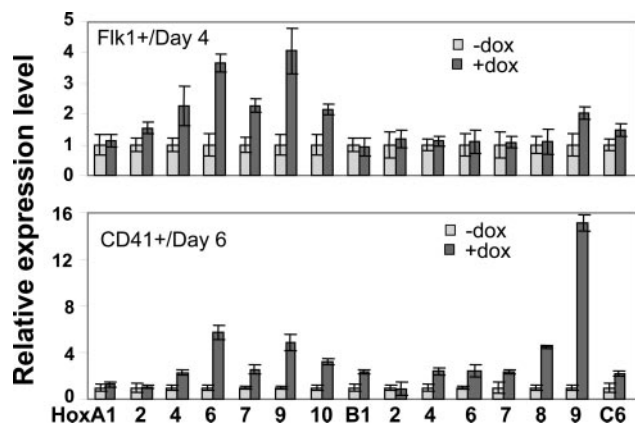
$\beta$ -actin and relative expression levels were derived with the comparative  $C_T$  method. For Fig. 2, probes labeled with FAM at the 5' end and TAMRA at the 3' end were purchased from Integrated

DNA Technologies. Multiplex reactions were performed with rodent GAPDH VIC-labeled probe/primer sets as normalization control (Applied Biosystems). Primer/probe sequences and PCR conditions were listed in Tables 2 and 3, which are published as supporting information on the PNAS web site.

**Cell Transplantation.** Six-week- to 3-month-old *Rag2*<sup>-/-</sup>/ *$\gamma$ c*<sup>-/-</sup> female mice were given two doses of 400 cGy  $\gamma$ -irradiation, separated by 4 h and were injected via lateral tail vein with  $2 \times 10^6$  cells in 400  $\mu$ l of IMDM/2% IFS. Transplanted mice were maintained under sterile conditions. Experiments were carried out with Institutional Animal Care and Use Committee approval.

**Spleen Colony Forming Assay.** Six- to 10-week-old *Rag2*<sup>-/-</sup>/ *$\gamma$ c*<sup>-/-</sup> female mice were irradiated with a single dose of 900 cGy  $\gamma$ -irradiation and  $10^5$  whole BM or  $10^6$  ESC-derived hematopoietic progenitor cells were administered retroorbitally in 200  $\mu$ l of PBS. An equal number of mice were irradiated and injected with PBS to control for host-derived spleen colonies. Mice were killed on different time points, and their spleens were fixed in Bouin's buffer and scored for the colony-forming units of the spleen (CFU-S).

**FACS Analysis.** Peripheral blood leukocytes, splenocytes, and bone marrow cells were treated with red cell lysis buffer (Sigma). Antibodies were purchased from Pharmingen BD Biosciences. Propidium iodide was added to exclude dead cells. Gr1<sup>+</sup>, B220<sup>+</sup>, or



**Fig. 2.** *Hox* gene expression profile in hematopoietic populations isolated from EBs by flow cytometry, determined by quantitative real-time RT-PCR analysis. (Upper) Flk1<sup>+</sup> cells from day 4 EBs with (+dox) or without (-dox) *Cdx4* induction from days 2 to 4 of EB differentiation. (Lower) CD41<sup>+</sup> cells from day 6 EBs with (+dox) or without (-dox) *Cdx4* induction from days 3 to 6 of EB differentiation.



CD3<sup>+</sup> cells were isolated either by FACS sorting or by positive selection with magnetic streptavidin-conjugated Dynabeads M280 (DynaL Biotech). The purity of sorted cells was verified by post-sorting FACS analysis.

**Genomic DNA Isolation and Southern Hybridization.** GFP and *HoxB4* probes were obtained separately by purification of an NcoI/Clal digested fragment from MSCV-ires-GFP and an EcoRI/XhoI fragment from MSCV-*HoxB4*-ires-GFP with a MinElu gel purification kit (Qiagen). Probes were labeled and Southern hybridization was performed according to standard protocols. Band intensity was measured by IMAGEQUANT.

## Results

**Cdx4 Expression Enhances Hemangioblast Formation.** The first hematopoietic cell to be detected in EBs is the hemangioblast, a bipotential precursor of hematopoietic and endothelial lineages (19, 22). Hemangioblasts are detected on days 3 and 4 of *in vitro* differentiation of ESCs into EBs, the time interval when *Cdx4* expression is highest (16). Therefore, we examined whether enforced expression of *Cdx4* could promote hemangioblast formation. To achieve conditional gene induction of *Cdx4*, a mouse *Cdx4* cDNA was cloned into the Ainv15 ESC line such that its expression is controlled by a tetracycline responsive promoter element (Fig. 6A, which is published as supporting information on the PNAS web site; 12). RT-PCR confirmed the induction of *Cdx4* after incubation of the cells for 24 h with tetracycline analogue doxycycline (Fig. 6B). Doxycycline was added to the EB differentiation media from day 2 to 3.2 (to allow maximal gene induction at 24 h, ref. 23) before blast colony assay in methylcellulose. Additionally, doxycycline was added to some methylcellulose cultures. Induction of *Cdx4* expression during EB development stimulated the formation of blast colonies, and the yield was further increased when *Cdx4* was continuously induced during methylcellulose culture (Fig. 1A). Individual blast colonies were picked, replated, and shown to generate both endothelial and hematopoietic progeny (Table 4 and Fig. 7, which are published as supporting information on the PNAS web site). *Cdx4* induction not only enhances formation of the hemangioblast, but appears to favor its differentiation toward hematopoietic fates.

***Cdx4* Promotes both Primitive and Definitive Hematopoiesis *In Vitro*.** To determine whether induction of *Cdx4* over a prolonged time interval might promote hematopoietic progenitor development in EBs, we incubated EBs with doxycycline from days 3 to 6 of differentiation and observed increased numbers of primitive erythroid and multipotential hematopoietic colonies (Fig. 1B). Cells expressing CD41 and c-kit, markers on early hematopoietic progenitors in both embryos and EBs (24–26), were also increased in day 6 EBs exposed to doxycycline (Fig. 1C), suggesting that *Cdx4* promotes hematopoietic colony formation by enhancing the specification, proliferation, or survival of clonogenic hematopoietic progenitors. Consistent with this finding, we demonstrated by using real-time RT-PCR that the expression level of hematopoietic-specific genes was elevated 2- to 3-fold in whole EBs after *Cdx4* activation between days 3 and 6 (Fig. 8, which is published as supporting information on the PNAS web site), suggesting that the enhanced gene expression by *Cdx4* reflects an increased percentage of hematopoietic cells in whole EBs. Of the genes we assayed,  *$\beta$ -H1*, *Tie2*, *LMO2*, *Scl*, and *GATA1* reflect both early hematopoietic development and definitive lineage differentiation, whereas  *$\beta$ -major*, *c-myb*, and *AML1* are markers of definitive hematopoiesis (reviewed in ref. 27). Elevated expression of these genes suggests that *Cdx4* activation promotes both primitive and definitive hematopoietic progenitor formation from differentiated ESCs.

OP9 is a stromal cell line derived from M-CSF deficient mice that supports the growth of hematopoietic progenitors (28). When EBs are dissociated and plated onto OP9, we typically observe only scant

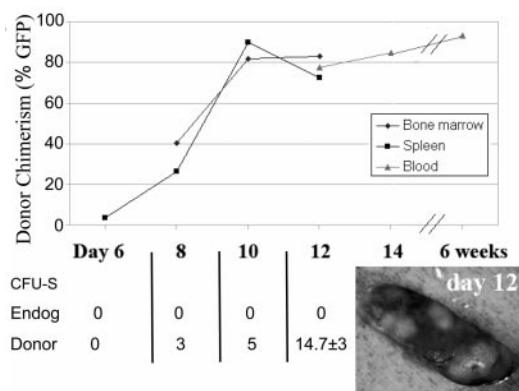
**Table 1. Surface antigen expression of ESC-derived cells growing on OP9 for 23 days**

Lineage	Surface marker	icdx4/+dox	ihoxB4/+dox
Myeloid	Gr-1	5.57	6.60
	Mac-1	52.17	28.31
Erythroid	Ter119	0.62	0.36
Lymphoid	CD4	0.10	0
	CD8	0	0
Progenitor/Meg HSC	B220	7.21	0.70
	CD41	85.94	89.94
	Scal	43.63	16.16
	c-kit	96.70	75.70
	c-kit/Scal	42.10	12.10
HSC/Endothelial	CD31	98.36	85.06
	CD34	92.22	10.25
	CD31/34	91.93	11.20
	Flk-1	0.31	0.26
	VE-Cadherin	71.73	51.23
Pan-hematopoietic	Endoglin	68.83	53.43
	CD45	85.32	50.40
	CD31/34/45	83.98	5.74

outgrowth of hematopoietic populations. However, induction of *Cdx4* enabled EB-derived hematopoietic progenitors to expand and undergo multilineage differentiation on OP9, as reflected by total cell counts (Fig. 1D) and flow cytometry with hematopoietic markers (Table 1). Compared to the gene expression profile of hematopoietic populations from day 6 EBs, expression of  $\beta$ -*H1* embryonic globin was significantly reduced, whereas the expression of  $\beta$ -*major*, the adult-type globin, was markedly elevated after coculture on OP9 stroma, indicating that *Cdx4* promotes maturation of definitive erythroid lineages (Fig. 1E).

Induction of *HoxB4* expression in EB-derived cells also promotes expansion of hematopoietic progenitors on OP9 stroma (12). Therefore, we compared the surface antigen and gene expression profile of OP9 cocultured cells expanded by either *Cdx4* or *HoxB4*. Of the lineage-specific surface antigens we assayed, cultures stimulated by *Cdx4* show a higher percentage of B220<sup>+</sup> cells, suggestive of enhanced lymphoid differentiation potential (Table 1). Expression of genes linked to B cell development was likewise increased in *Cdx4*-induced OP9 cocultured cells (Fig. 1*F*). By surface antigen profile, >76% of *Cdx4*-expanded cells displayed surface antigen features comparable to definitive hematopoietic progenitors derived from the aorta-gonad-mesonephros region of the embryo, namely coexpression of CD41<sup>+</sup>/CD31<sup>+</sup>/CD34<sup>+</sup>/c-kit<sup>+</sup> (29–31). They likewise appear to acquire CD45 expression during maturation *in vitro*. By contrast, cells stimulated to proliferate on OP9 by *HoxB4* expression had lower levels of CD34 and CD45. CD45 is a pan-hematopoietic marker that is developmentally regulated, appearing on mature hematopoietic populations after activation of CD41 in both the embryo and EBs (24, 25). Our observation of a higher percentage of CD45<sup>+</sup> cells in our *Cdx4*-expanded EB-derived populations suggests that *Cdx4* and culture on OP9 stroma promotes the switch from primitive to definitive hematopoiesis more efficiently than *HoxB4*. CD34 is developmentally and functionally regulated, and its expression is influenced by the activation state of stem cells (32, 33). Higher expression of CD34<sup>+</sup> in *Cdx4*-expanded cells suggests that these cells are in an actively cycling state, consistent with the rapid proliferation of the cultures.

**Cdx4 Induction Modulates Hox Gene Expression in Hematopoietic Cells.** Genetic and molecular biological studies in *Drosophila*, zebrafish, and mouse have established that the Caudal-related family of homeodomain transcription factors regulates *Hox* gene expression patterns (reviewed in ref. 13). To explore the *Hox* gene expression profiles that result from *Cdx4* induction during hematopoietic commitment in EBs, we performed real-time RT-PCR



**Fig. 3.** Donor cell chimerism and CFU-S formation in mice engrafted with *cdx4/hoxB4*-modified cells. Mice were killed from days 6 to 12 and their spleens analyzed for CFU-S formation; the picture (Lower Right) shows a representative d12 spleen after fixation.

analysis on the expression of *Hox A, B, and C* cluster genes in Flk1<sup>+</sup> day 4 EB cells and CD41<sup>+</sup> day 6 EB cells. *Cdx4* induction resulted in enhanced expression of posterior *Hox* genes (*A6, A7, A9, A10, B9, and C6*) in these hematopoietic populations (Fig. 2). Comparable induction was not observed in the nonhematopoietic Flk1<sup>+</sup> fraction of cells (data not shown). These data suggest that *Cdx4* promotes blood formation by influencing *Hox* gene patterning during hematopoietic mesoderm commitment.

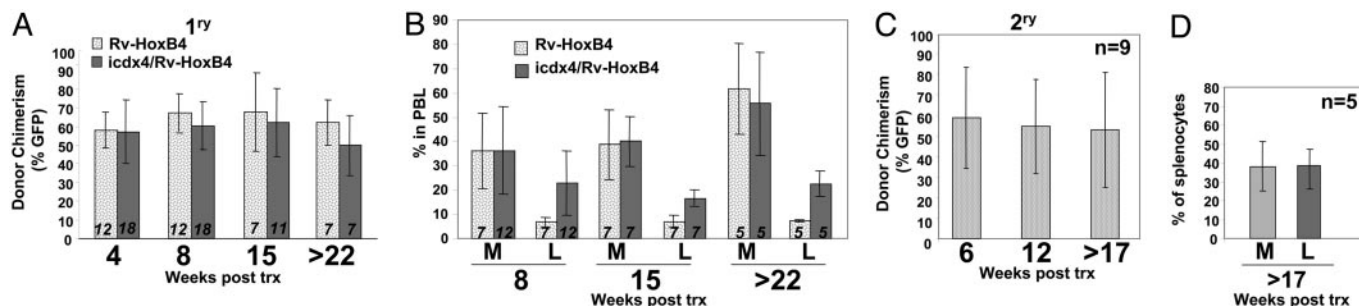
#### ***Cdx4* Enables Engraftment of ES-Derived Hematopoietic Progenitors.**

We next explored whether *Cdx4* enables engraftment of ES-derived hematopoietic progenitors in lethally irradiated mice (schema in Fig. 9A, which is published as supporting information on the PNAS web site). Contrary to our expectations, hematopoietic populations derived from *Cdx4*-induced EBs protected only a minority of mice (8 of 30) from radiation-induced bone marrow aplasia. Donor chimerism in surviving mice was low (average <1%, Fig. 9B), suggesting that the transplanted population contained only small numbers of definitive HSCs or was comprised of progenitors with limited self-renewal potential. We noted that *Cdx4* induction in EBs increased *HoxB4* expression only 2-fold and that OP9 cocultured cells expanded by *HoxB4* induction (or retroviral transduction of *HoxB4*) expressed significantly more *HoxB4* than cells expanded by *Cdx4* (Fig. 2 and data not shown). The weak enhancement of *HoxB4* expression by *Cdx4* appears inadequate to maintain or expand transplantable HSCs on OP9 stromal cultures. Given that *HoxB4* is a major factor in the self-renewal and expansion of ESC-derived

HSCs, we examined whether combining the hematopoietic specification ability of *Cdx4* with the self-renewal potential of *HoxB4* could improve engraftment of hematopoietic populations derived from differentiated ESCs. EBs were formed from the conditional *Cdx4* cell line. Some cultures were left uninduced, whereas others were induced by doxycycline during days 3 to 6 of EB development. At day 6, EB cells from both sets of cultures were transduced with a retroviral vector expressing *HoxB4* linked via internal ribosomal entry site (ires) to green fluorescent protein (GFP) and subsequently cultured on OP9 stromal cells for 10–14 days (under our present conditions, OP9 coculture appears to be a necessary step before transplantation; see also ref. 12). Cultured cells were then injected intravenously into lethally irradiated lymphocyte/NK cell-deficient *Rag2*<sup>-/-</sup>/*γc*<sup>-/-</sup> mice (34).

A cohort of animals injected with 10<sup>6</sup> *Cdx4-HoxB4* modified cells were killed at different time intervals and examined for evidence of short-term hematopoietic chimerism in the bone marrow, spleen, and peripheral blood. Within 8–12 days, animals developed high levels of GFP<sup>+</sup> cells in all hematopoietic tissues tested and showed characteristic splenic hematopoietic colonies (CFU-S, Fig. 3). Although not the equivalent of the long-term HSC (35), the CFU-S reflects a primitive multipotent myeloid progenitor that previously has not been demonstrated reliably in animals engrafted from ESCs differentiated *in vitro*. The frequency of CFU-S detectable in stromal cocultures (14.7 ± 3 in 10<sup>6</sup> cells) is ≈10-fold less than whole bone marrow (data not shown). These data suggest that EB cells expanded on OP9 stromal cocultures produce hematopoietic progenitors that support rapid engraftment after radiation-induced marrow aplasia.

In data from three independent transplantation experiments with cells genetically modified by either *HoxB4* alone, or both *Cdx4* and *HoxB4*, survival due to the radioprotective effect of transplanted cells was close to 100% at 8 weeks (12 of 13 for *HoxB4*; 18 of 18 for *Cdx4/HoxB4*). Flow cytometric monitoring of GFP<sup>+</sup> cells in the peripheral blood of transplanted animals showed high-level donor chimerism that was stable over at least 6 months (Fig. 4A). Moreover, myeloid, lymphoid, and erythroid lineages were reconstituted in the peripheral blood, spleen, lymph nodes, bone marrow, and thymus of engrafted mice (Fig. 4B; see also Fig. 10A–C, which is published as supporting information on the PNAS web site; see also ref. 36). Interestingly, when compared with mice transplanted with cells treated with *HoxB4* alone, mice engrafted with *Cdx4/HoxB4*-treated cells consistently showed a higher degree of lymphoid reconstitution (Fig. 4B and 10A–C), a result that correlated with the enhanced percentage of B220<sup>+</sup> cell formation in OP9 cultures (Table 1). Bone marrow from primary animals engrafted with *Cdx4/HoxB4*-expressing cells successfully reconstituted multiple lineages of hematopoietic cells when transplanted into lethally



**Fig. 4.** Donor cell chimerism and multilineage engraftment in irradiated primary and secondary mice. (A) Donor chimerism (%GFP<sup>+</sup>) in peripheral blood of mice engrafted with *HoxB4* or *Cdx4/HoxB4* modified hematopoietic populations differentiated from ESCs >22 weeks after transplantation. (B) Flow cytometry analysis of peripheral blood cells expressing either myeloid antigens (Gr-1, M) or lymphoid antigens (CD3/B220, L). Number of mice analyzed at each time point is indicated. (C) Donor chimerism in peripheral blood of secondary animals. Bone marrow (BM) from primary recipients engrafted at least 12 weeks was transplanted into secondary recipients. (D) Myeloid-lymphoid reconstitution of splenocytes from secondary animals. Error bars represent standard deviation. 1<sup>ry</sup>, primary; 2<sup>ry</sup>, secondary.





*HoxB4*, *B8*, and *B9*; refs. 40–42). *Cdx4* overexpression can rescue blood progenitor formation in ESCs that are deficient in *Mill*, a *Hox* gene regulator involved in definitive hematopoiesis (26, 43, 44). Cluster C *Hox* genes such as *C6*, whose expression is also enhanced by *Cdx4* activation, are particularly linked to lymphoid development (45–47). Given the role of *Cdx* genes in patterning of posterior tissues during embryogenesis, we conclude that *Cdx4* is acting to enhance mesodermal commitment to hematopoietic fates through modulation of the *Hox* code. No significant defects in hematopoiesis were observed in *Cdx1* and/or *Cdx2* knockout mice, with the exception that yolk sac circulation is abnormal in *Cdx2*-deficient embryos (14, 15). However, given the reports of *Cdx2* involvement in human and murine leukemogenesis (17, 18), it is likely that there are overlapping and, perhaps, redundant roles of the *Cdx* genes in hematopoiesis.

Previously, we showed that expression of *HoxB4* in differentiating ESCs or primitive yolk sac progenitors enabled engraftment of irradiated mice, but the recipient animals showed only low levels of lymphoid reconstitution (12). Despite initial reports that retroviral transduction of bone marrow with *HoxB4* produced HSC expansion and enhanced competitive engraftment without distortion of hematopoietic differentiation (5–7), several groups have now observed alterations in the lympho-myeloid differentiation program (9, 11, 49). We conclude that *HoxB4* can compromise lymphoid engraftment, because the predominant lymphocyte populations in our engrafted animals lack GFP expression, which we have shown correlates with the transcriptional silencing of the *HoxB4* provirus (see Fig. 12, which is published as supporting information on the PNAS web site). Current efforts are underway to derive HSCs from ESCs without ectopic *HoxB4* gene expression, and evidence exists from one study that *HoxB4* is dispensable for generating hematopoiesis from human (48).

The self-renewing, multipotential nature of the HSC was demonstrated definitively in the mid-1980s in experiments that used

retroviruses as unique genetic markers to trace HSC fates following bone marrow transplantation (37, 38). The demonstration that highly purified lymphoid and myeloid blood cells in engrafted mice showed common sites of proviral integration established that multiple blood lineages derived from single precursor cells. Some of these clones were detected again in the hematopoietic tissue of secondary recipient mice (38, 39). The evidence that single clones can reconstitute the lympho-myeloid system of both primary and secondary recipients established the paradigmatic definition of stem cells as self-renewing multipotential progenitors. In this study, we applied classical Southern hybridization analysis of proviral integration sites in engrafted blood lineages of primary and secondary mice to demonstrate the clonal derivation of HSCs from murine ESCs. Long-term reconstitution of primary and secondary mice with common clones demonstrates self-renewal, whereas evidence that myeloid and lymphoid cells derive from common clones demonstrates multilineage differentiation potential. Taken together, our data validate the classical definition of a self-renewing, multilineage hematopoietic stem cell and indicate the successful derivation of long-term HSCs from ESCs *in vitro*. The application of similar principles to the derivation of HSCs from human ESCs, coupled to methods to generate genetically matched ESCs by nuclear transfer, provides an important theoretical foundation for combined cell and gene therapy for the treatment of genetic and malignant disorders of the blood (3).

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